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# Monoclonal Anti-CD18 Antibody Prevents Transcellular Biosynthesis of Cysteinyl Leukotrienes In Vitro and In Vivo and Protects Against Leukotriene-Dependent Increase in Coronary Vascular Resistance and Myocardial Stiffness

Angelo Sala, PhD; Giuseppe Rossoni, MS; Ferruccio Berti, PhD; Carola Buccellati, PhD; Albino Bonazzi, MS; Jacques Macclouf, PhD†; Giancarlo Folco, PhD

**Background**—Cysteinyl leukotrienes (cys-LT) can constrict small and large vessels and increase vascular permeability. Formation of cys-LT arising from polymorphonuclear leukocytes (PMNL) and endothelial cell cooperation (transcellular synthesis) led to the hypothesis that PMNL–endothelial cell adhesion may represent a key step toward the formation of vasoactive cys-LT.

**Methods and Results**—We studied the effect of pretreatment with a monoclonal antibody directed against the CD18 subunit of PMNL  $\beta_2$ -integrin on the synthesis of cys-LT in a PMNL-perfused isolated rabbit heart in vitro and in a model of permanent ligation of the left descending coronary artery in the rabbit in vivo. Challenge of PMNL-perfused rabbit hearts with formyl-met-leu-phe ( $0.3 \mu\text{mol/L}$ ) caused synthesis of cys-LT and increase in coronary perfusion pressure that were prevented by the anti-CD18 antibody. Similar results were obtained with the use of A-23187 ( $0.5 \mu\text{mol/L}$ ) as a challenge. Persistence of PMNL-associated myeloperoxidase activity in the perfusion buffer was observed in the presence of the anti-CD18 antibody, indicating decreased PMNL infiltration. Coronary artery ligation in vivo increased urinary excretion of leukotriene  $E_4$ , supporting the activation of the 5-lipoxygenase pathway during experimental acute myocardial infarction. Pretreatment with the anti-CD18 antibody ( $1 \text{ mg/kg}$ ) prevented the increase in leukotriene  $E_4$  excretion.

**Conclusions**—These data support the importance of adhesion in promoting cys-LT formation, originating from PMNL–endothelial cell cooperation, and contributing to myocardial stiffness and increased coronary resistance. (*Circulation*. 2000;101:1436-1440.)

**Key Words:** leukocytes ■ endothelium ■ cell adhesion molecules ■ prevention

Adherence of polymorphonuclear leukocytes (PMNL) to vascular endothelial cells (EC) is one of the earliest steps in inflammation in general and in the pathogenesis of ischemia-reperfusion tissue injury.<sup>1</sup> PMNL adherence to EC involves several adhesion molecules that mediate cell-cell interactions and orchestrate a complex series of events.<sup>2</sup> The activation of adherent leukocytes in response to various stimuli characterizes the next step in an acute inflammatory response involving diapedesis, release of proinflammatory lipid mediators, alterations in vessel tone, and vascular leakage. Several structurally diverse lipid molecules derived from arachidonic acid are synthesized during inflammatory reactions in vivo; among them, leukotrienes have attracted considerable interest.<sup>3</sup>

Leukotriene  $C_4$ ,  $D_4$ , and  $E_4$  (cys-LT) are potent vasoactive mediators that constrict small and large vessels and modify cardiac and coronary functions, the microcirculation, and some of the manifestations of ischemia-reperfusion injury.<sup>4,5</sup> Additionally, they have vasopermeant properties that might be of relevance for the extravasation of leukocytes from the vessel lumen to the tissue.<sup>6</sup> Their generation exhibits remarkable cellular specificity; however, cys-LT formation also may occur through transfer of reactive intermediates between adjacent cells, which represents a specialized mode of cell-cell communication.<sup>7</sup> PMNL-platelet interactions involving the lipoxygenase pathway, which may be important in hemostasis and inflammation, were first documented by Marcus et al.<sup>8</sup> More recently, cooperation of donor PMNL with acceptor

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†On July 14, 1998, during this investigation, Dr J. Macclouf died after having a stroke. None of this work would have been possible without his extraordinary knowledge of the biochemistry of eicosanoids. We wish to dedicate this work to his memory.

The Methods section of this article can be found at <http://www.circulationaha.org>

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EC in processing the reactive intermediate  $\text{LTA}_4$  into biologically active  $\text{LTC}_4$  has been demonstrated.<sup>9</sup> This process has been termed “transcellular biosynthesis” and suggests that the cellular environment (ie, cell-cell interactions) represents an important control mechanism in the production of eicosanoids, which may ultimately affect organ function.<sup>10</sup> Indeed, challenge of PMNL within the coronary vasculature causes coronary vasoconstriction<sup>11</sup> associated with PMNL extravasation and widespread perivascular edema,<sup>12</sup> both dependent on endogenous cys-LT formation. PMNL-EC adhesion is regulated by several cell-surface adhesion molecules; among them PMNL  $\beta_2$ -integrins are known to play a significant role in firm adhesion of PMNL to EC.<sup>2</sup>

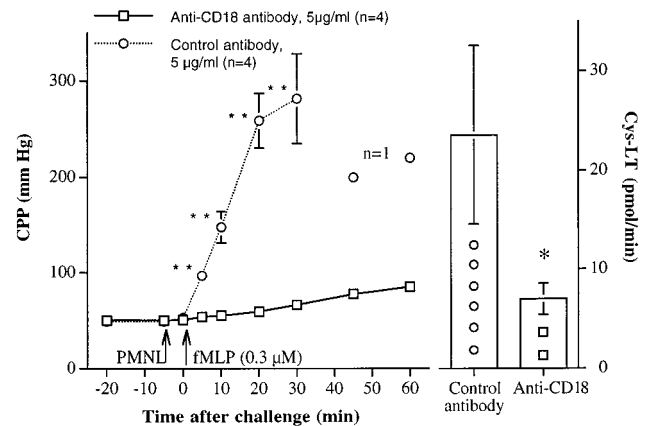
In the present study, we provide evidence that a monoclonal antibody (mAb) directed against the CD18 subunit of PMNL  $\beta_2$ -integrins (a) inhibits cys-LT generation decreasing PMNL-dependent tissue edema and coronary resistance in the isolated heart of the rabbit in vitro and (b) inhibits the increased urinary leukotriene excretion occurring after acute myocardial infarction of the left ventricular wall in the rabbit in vivo.

## Results

Intravascular challenge of granulocyte macrophage-colony stimulating factor (GM-CSF)-primed PMNL in the isolated perfused heart with the chemotactic peptide formyl-met-leu-phe (fMLP,  $0.3 \mu\text{mol/L}$ ) in the presence of a murine nonbinding mAb (MOPC-21,  $5 \mu\text{g/mL}$ ) resulted in a significant increase of coronary resistance to perfusion (coronary perfusion pressure, CPP), causing the arrest in systole in 3 of 4 isolated hearts within 30 to 45 minutes after challenge. Basal left ventricular end-diastolic pressure (LVEDP) values were very stable ( $5 \pm 0.2 \text{ mm Hg}$ ,  $n=4$ ) and increased markedly after challenge (at 20 minutes,  $55 \pm 14.6 \text{ mm Hg}$ ,  $n=4$ ,  $P<0.01$  vs basal).

High-performance liquid chromatography (HPLC) analysis of the total volume of the circulating perfusate (44 to 47 mL) collected at the end of the experiment allowed positive identification of cys-LT by on-line UV-spectrum analysis. Pretreatment with the anti-CD18 antibody (6.5E,  $5 \mu\text{g/mL}$ ) resulted in a significant inhibition of the increase in coronary perfusion pressure (CPP), allowing survival of all isolated hearts throughout the observation period of 60 minutes, and was accompanied by a significant decrease in cys-LT formation (Figure 1). LVEDP values did not differ from basal values ( $5 \pm 0.2 \text{ mm Hg}$ ,  $n=4$ ). The assay of cell-associated myeloperoxidase (MPO) enzyme activity in the recirculating buffer confirmed a rapid disappearance of MPO in the presence of control mAb, whereas pretreatment with the anti-CD18 mAb resulted in a significantly inhibited adhesion of PMNL (Figure 2).

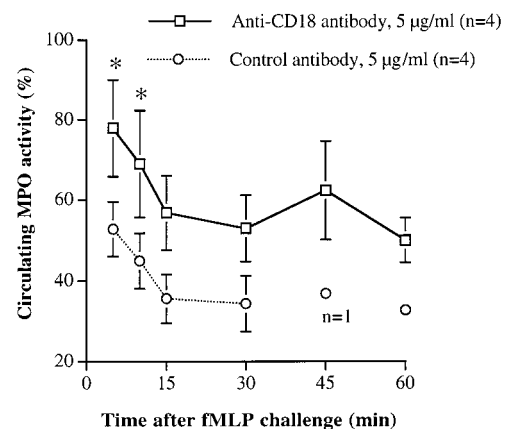
To test whether the observed effect of the anti-CD18 antibody could be reversed by a more sustained activation of the 5-lipoxygenase (5-LO), PMNL-perfused, isolated hearts were challenged with A-23187 ( $0.5 \mu\text{mol/L}$ ). As previously reported,<sup>11</sup> challenge with A-23187 induced the PMNL-dependent formation of cys-LT, together with a significant increase in CPP, which resulted in arrest in systole in 3 of 4 isolated hearts within 20 to 30 minutes after challenge. As



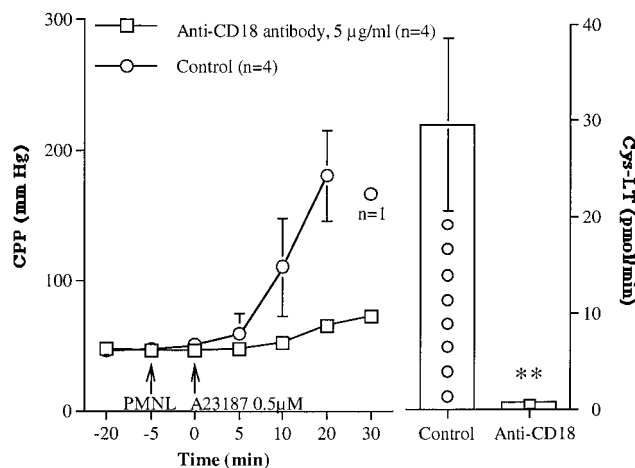
**Figure 1.** Effect of treatment with anti-CD18 mAb on PMNL-dependent cys-LT formation and changes in coronary resistance on challenge with fMLP in rabbit isolated heart. Isolated rabbit hearts were perfused with purified human PMNL ( $10^7$  cells), primed with GM-CSF ( $1 \text{ nmol/L}$ , 30 minutes), and challenged with fMLP ( $0.3 \mu\text{mol/L}$ ) in presence of anti-CD18 mAb ( $6.5\text{E}$ ,  $5 \mu\text{g/mL}$ ,  $\square$ ) or of isotype-matched, nonbinding control mAb (MOPC-21,  $5 \mu\text{g/mL}$ ,  $\circ$ ). CPP (left) was monitored continuously; formation of leukotrienes (right) was evaluated by reverse-phase HPLC of entire volume of recirculating perfusate. Values are expressed as mean  $\pm$  SEM ( $n=4$ ). \* $P<0.05$ , \*\* $P<0.01$  vs control.

observed with fMLP, pretreatment with the anti-CD18 mAb significantly reduced the increase in CPP, and all isolated hearts survived throughout the observation period of 30 minutes (Figure 3, left) and resulted in a marked suppression of the formation of cys-LT (Figure 3, right), suggesting the pivotal role of adhesion in their production.

The assay of circulating PMNL provided evidence of efficacy of the pretreatment with the anti-CD18 antibody in inhibiting PMNL adhesion. After A-23187 activation, a rapid disappearance of PMNL from the recirculating buffer was observed, suggesting intravascular adhesion. However, pre-



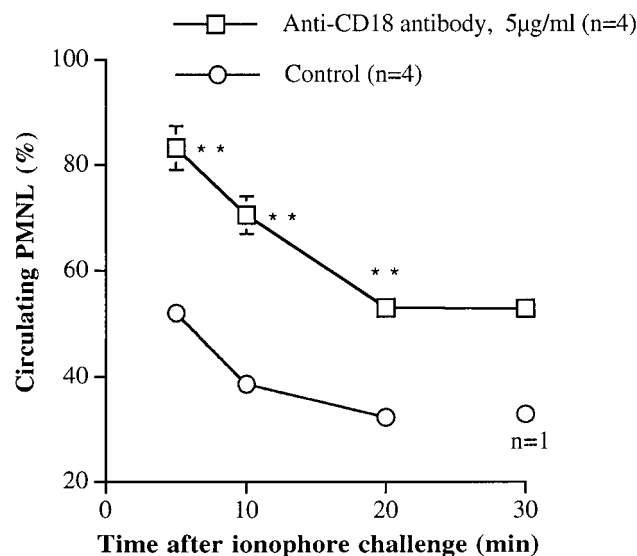
**Figure 2.** Effect of treatment with anti-CD18 mAb on circulating PMNL after challenge with fMLP. Isolated rabbit hearts were perfused with purified human PMNL ( $10^7$  cells), primed with GM-CSF ( $1 \text{ nmol/L}$ , 30 minutes), and challenged with fMLP ( $0.3 \mu\text{mol/L}$ ) in presence of anti-CD18 mAb ( $6.5\text{E}$ ,  $5 \mu\text{g/mL}$ ,  $\square$ ) or of isotype-matched, nonbinding control mAb (MOPC-21,  $5 \mu\text{g/mL}$ ,  $\circ$ ). PMNL-associated myeloperoxidase activity was evaluated in aliquots of recirculating perfusate taken at 5, 10, 20, 30, 45, and 60 minutes after challenge. Values are expressed as percent  $\pm$  SEM ( $n=4$ ) of MPO activity evaluated immediately before challenge with fMLP. \* $P<0.05$  vs control.



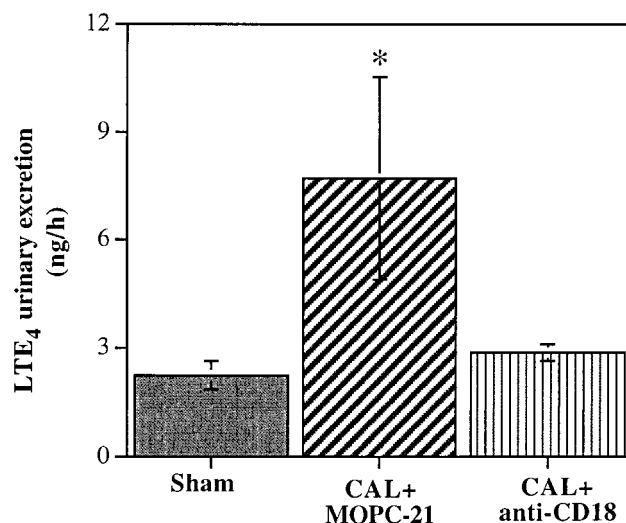
**Figure 3.** Effect of treatment with anti-CD18 mAb on PMNL-dependent cys-LT formation and changes in coronary resistance on challenge with A23187 in rabbit isolated heart. Isolated rabbit hearts were reperused with purified human PMNL ( $5 \times 10^6$  cells) and challenged with A23187 ( $0.5 \mu\text{mol/L}$ ) in presence ( $\square$ ) or absence ( $\circ$ ) of pretreatment with anti-CD18 mAb (6.5E,  $5 \mu\text{g/mL}$ ). CPP (left) was monitored continuously; formation of leukotrienes (right) was evaluated by reverse-phase HPLC of the entire volume of recirculating perfusate. Values are expressed as mean  $\pm$  SEM ( $n=4$ ).  $**P<0.01$  vs control.

treatment with the anti-CD18 mAb resulted in persistence of PMNL-associated MPO activity in the recirculating buffer as a result of inhibited adhesion (Figure 4).

Challenge with fMLP ( $0.3 \mu\text{mol/L}$ , 60 minutes) of GM-CSF-primed PMNL preparations in suspension showed a substantial release of  $\text{LTA}_4$  metabolites, which was not affected by pretreatment with anti-CD18 mAb 6.5E



**Figure 4.** Effect of treatment with anti-CD18 mAb on circulating PMNL after challenge with A23187. Isolated rabbit hearts were reperused with purified human PMNL ( $5 \times 10^6$  cells) and challenged with A23187 ( $0.5 \mu\text{mol/L}$ ) in presence ( $\square$ ) or absence ( $\circ$ ) of pretreatment with anti-CD18 mAb (6.5E,  $5 \mu\text{g/mL}$ ). PMNL-associated MPO activity was evaluated in aliquots of recirculating perfusate taken at 5, 10, 20, and 30 minutes after challenge. Values are expressed as percent  $\pm$  SEM ( $n=4$ ) of MPO activity evaluated immediately before challenge with A23187.  $**P<0.01$  vs control.



**Figure 5.** Urinary excretion of  $\text{LTE}_4$  after heart surgery in rabbits. Bladder was manually evacuated at beginning and 3 hours after surgery to evaluate actual excretion of  $\text{LTE}_4$  after sham operation or CAL. Coronary artery-ligated rabbits were either treated with anti-CD18 mAb (6.5E,  $1 \text{ mg/kg IV}$ ) or with isotype-matched, nonbinding control mAb (MOPC-21,  $1 \text{ mg/kg IV}$ ) 30 minutes before heart surgery and CAL.  $\text{LTE}_4$  was evaluated by enzyme immunoassay on immunofiltration extraction. Values are expressed as mean  $\pm$  SEM of total excretion of  $\text{LTE}_4$  ( $\text{ng/h}$ ).  $*P<0.05$  vs sham operated.

( $25.5 \pm 3.4$  vs  $31.1 \pm 2.9 \text{ pmol}/10^6 \text{ PMNL}$  in control and anti-CD18-treated cells, respectively;  $n=3$ ). Similarly, production of  $\text{LTA}_4$  metabolites on challenge with A-23187 ( $0.5 \mu\text{mol/L}$ , 30 minutes) was not affected by pretreatment with the anti-CD18 mAb ( $5 \mu\text{g/mL}^{-1}$ ) ( $291.3 \pm 13.7$  vs  $265.3 \pm 10.3 \text{ pmol}/10^6 \text{ PMNL}$  in control and anti-CD18-treated cells, respectively;  $n=3$ ).

### In Vivo Studies

Excretion of  $\text{LTE}_4$  in urine was evaluated during the 3 hours after permanent ligation of the left descending coronary artery (coronary artery ligation, CAL) in the rabbit, resulting in acute myocardial infarction of the left ventricular wall, and was compared with the values obtained in sham-operated animals. Urinary excretion of  $\text{LTE}_4$  was significantly higher in the CAL group, treated with the nonbinding IgG1 mAb MOPC-21 ( $1 \text{ mg/kg IV}$ , 15 minutes before ligation), indicating endogenous production of cys-LT during the ischemia associated with the coronary ligation. Treatment with the anti-CD18 mAb 6.5E ( $1 \text{ mg/kg IV}$ , 15 minutes before ligation) fully prevented the increase in  $\text{LTE}_4$  excretion (Figure 5).

### Discussion

In the present study, we report that a mAb against the functional epitopes of leukocyte CD18 complex of adhesive glycoproteins prevents the generation of cys-LT taking place through the interaction of PMNL with coronary EC. Cell adherence may therefore represent an important mechanism regulating leukotriene generation in situ and is in line with our current understanding of cys-LT as paracrine hormones.

Neither PMNL nor EC can synthesize cys-LT from the precursor AA; however, the former have been shown to



produce predominantly  $\text{LTA}_4$ ,<sup>13</sup> whereas the latter possess a remarkably effective metabolic capacity for cys-LT from the epoxide precursor  $\text{LTA}_4$ . It is therefore likely that during adhesion, a privileged interface between the donor PMNL and the acceptor EC is formed, creating the necessary conditions to transfer the unstable intermediate  $\text{LTA}_4$ .  $\text{LTC}_4$ -activated endothelium may then become adhesive for PMNL through the surface expression of platelet-activating factor<sup>14</sup> and P-selectin, providing a self-amplifying loop that may result in increased transcellular synthesis of cys-LT.

The mechanism that explains the increase in coronary vascular resistance and myocardial stiffness involves activated PMNL attaching to the vascular endothelium and triggering transcellular biosynthesis of cys-LT; local formation of cys-LT results in edema formation and extravascular compression of coronary microvessels, as previously shown by scanning electron microscopy.<sup>12</sup> Local production of cys-LT also may contribute to active coronary vasoconstriction; in fact, the increase in coronary perfusion pressure evoked by PMNL activation is partially reversible after intracoronary injection of sodium nitroprusside.<sup>11</sup> The inhibition of PMNL-EC adhesion by the anti-CD18 mAb, reducing cell-cell contact and making transcellular biosynthesis events much less efficient, exerts protective effects against cardiac inflammation and its functional outcomes.

We used the presence of cell-associated MPO activity as an indirect tool to quantitatively evaluate the extent of PMNL adhesion to its target cells and obtained evidence that anti-CD18 mAb effectively blunted PMNL sequestration through the coronary bed. A significant body of evidence supports the notion that the inflammatory tissue damage that accompanies ischemia or ischemia-reperfusion is mediated to a large extent by PMNL.<sup>15</sup> Accordingly, prevention of leukocyte-EC interaction, through the use of mAbs directed against adhesion molecules, has proven successful in limiting ischemic damage in experimental models.<sup>16,17</sup> A study with isolated PMNL-glomerular EC cocultures showed that transcellular synthesis of cys-LT was inhibited by pretreatment with an anti-CD18 mAb.<sup>18</sup> Our work extends these findings to a functional organ system and provides a link between adhesion of PMNL, synthesis of cys-LT, and functional modifications.

The model of in vitro PMNL-dependent cardiac damage used for this study is different from more complex in vivo models of ischemia-reperfusion injury. Recently, a 54% reduction in PMNL accumulation and a 57% decrease of myocardial necrosis after ischemia-reperfusion was observed in CD18-deficient mice and intracellular adhesion molecule-1-deficient mice,<sup>19</sup> supporting a critical role of these cell adhesion molecules in myocardial cell injury of the reperfused myocardium. The more significant functional protection observed in our study (>80% inhibition of increase in CPP and LVEDP) is not unexpected given the fact that our model is uniquely PMNL dependent, whereas it is conceivable that in vivo other cells and factors may contribute to the development of the cardiac injury.

Measurement of  $\text{LTE}_4$  in urine has been largely adopted as a noninvasive, time-integrated index of cysteinyl leukotriene synthesis in vivo. Evaluation of urinary  $\text{LTE}_4$  excretion

showed a significant increase after permanent coronary ligation in the rabbit, in agreement with the results of 2 independent groups reporting increased urinary  $\text{LTE}_4$  excretion in patients with coronary artery disease and in patients after myocardial infarction.<sup>20,21</sup> The observed inhibition after pretreatment with anti-CD18 antibody supports the hypothesis that pathophysiologically relevant cys-LT formation within an ischemic myocardium may represent the outcome of transcellular biosynthetic events.

The increased urinary excretion of  $\text{LTE}_4$  associated with the CAL observed in the present study is also clearly complementary with our previous results with the same model, in which we showed a significant decrease of the mortality rate by pretreatment with a specific leukotriene synthesis 5-lipoxygenase-activating protein (FLAP) inhibitor.<sup>22</sup>

Although it may seem difficult to hypothesize the infiltration of neutrophils into the infarcted area in the time course described, we must point out that under our working hypothesis there is no need to have actual neutrophil infiltration, but it would be sufficient for them to adhere to EC to achieve local leukotriene formation associated with altered vascular permeability and tone. In fact, it has recently been shown that increased endothelial permeability occurs even in the absence of neutrophil infiltration.<sup>23</sup>

The leukocyte count has been originally proposed as a valuable routine index for the assessment of risk for myocardial infarction.<sup>24</sup> Since then, a number of epidemiological studies have shown the existence of a significant relation between blood white cell count and the occurrence of coronary heart disease (eg, angina pectoris and myocardial infarction).<sup>25</sup> Furthermore, enhanced neutrophil expression of CD11b/CD18 adhesion receptors has been recently reported in patients with unstable angina.<sup>26</sup> Our data, supporting the functional relevance of CD18-mediated, PMNL-dependent synthesis of cys-LT, provide a link between PMNL implication in the natural history of coronary heart disease and increased urinary  $\text{LTE}_4$  levels in patients with cardiac ischemia, two observations apparently uncorrelated.

In conclusion, we propose that among the PMNL-dependent factors contributing to the development of cardiac damage associated with ischemia, the production of cys-LT through transcellular biochemical mechanisms may have a significant role and may represent a potential therapeutic target.

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